

# Germline-Activating Mutation in the Kinase Domain of *KIT* Gene in Familial Gastrointestinal Stromal Tumors

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**The proto-oncogene *KIT* encodes the receptor tyrosine kinase KIT. Gain-of-function mutations in the juxtamembrane domain of *KIT* have been reported in human gastrointestinal stromal tumors. In a family with multiple gastrointestinal stromal tumors and diffuse hyperplasia of myenteric plexus layer, we have identified another mutation of *KIT*, a single base mutation, resulting in the substitution of Glu for Lys<sup>642</sup> in the kinase I domain, and studied its biological effect in a cellular system. The mouse homologue of the human *KIT* mutant was generated by site-directed mutagenesis and stably transfected into the interleukin-3-dependent Ba/F3 murine cell line. The oncogenic potential of the mutated *KIT* was assessed *in vitro* by a proliferation assay and *in vivo* by transplantation into nude mice. Transfected Ba/F3 cells grew autonomously in absence of growth factors and formed tumors in nude mice. Substitution of Glu for Lys<sup>642</sup> is an oncogenic mutation in the tyrosine kinase domain of *KIT*. As germline heterozygous mutation, it causes a diffuse hyperplasia of myenteric interstitial cells of Cajal during embryonic development and occurrence of multiple gastrointestinal stromal tumors at adulthood. (Am J Pathol 2000, 157:1581–1585)**

The *KIT* proto-oncogene is the cellular homologue of the oncogene *v-kit* present in the genome of Hardy-Zuckerman 4-feline sarcoma virus.<sup>1</sup> It encodes the transmembrane receptor tyrosine kinase KIT that is member of the same subfamily as the receptors for platelet-derived growth factor and for colony-stimulating factor 1.<sup>2,3</sup> KIT consists of an extracellular domain with five immunoglobulin-like repeats, a transmembrane domain, a juxtamembrane domain, and a kinase domain separated into two

parts (I and II) by an insert.<sup>2–4</sup> The ligand for KIT is stem cell factor (SCF).<sup>5</sup> The SCF-KIT system plays crucial roles for the development of melanocytes, erythrocytes, germ cells, mast cells,<sup>4</sup> and interstitial cells of Cajal (ICCs).<sup>6</sup> ICCs are mesenchymal cells, closely associated with nerves and smooth muscle cells of the gastrointestinal (GI) tract.<sup>7</sup> Absence of the SCF-KIT signaling pathway causes the lack of ICCs surrounding the myenteric plexus (ICCs-MP) and the lack of pacemaker component (electrical slow waves) in stomach and intestine.<sup>8,9</sup>

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the human GI tract. A majority of GISTs are KIT-immunoreactive<sup>10,11</sup> and several mutations in the juxtamembrane domain of *KIT* have been reported in GISTs. These mutations cause a constitutive, ligand-independent, activation of KIT responsible for their oncogenic potential.<sup>10,12</sup> Furthermore, a germline mutation in the juxtamembrane domain of *KIT* gene has been identified in familial and multiple GISTs.<sup>13</sup> We have studied a family with multiple GISTs and diffuse hyperplasia of ICCs-MP in the gut unaffected by GISTs. No mutation was found in the juxtamembrane domain, but a novel mutation in the kinase domain I was identified, and we further investigated the functional aspects of this mutant.

## Materials and Methods

### Patients

A French woman and her son (67 and 40 years old, respectively) presented with multiple (20 and 13 tumors, respectively) macroscopic GISTs, measuring from 1 to 8 cm, in duodenum and jejunum. All tumors examined were of low malignancy grade (ie, mitotic index <1 mitosis per

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10 high-power fields) and in both cases there was no metastasis. There was no familial history of von Recklinghausen's complex, multiple endocrine neoplasia, or intestinal ganglioneuromatosis. A detailed report of the cases will appear elsewhere. Tissues from GISTs, intestinal mucosa unaffected by GISTs, and normal pancreatic tissues were obtained from both patients as surgical waste at the time of pancreato-duodenectomy performed to relieve intestinal obstruction.

### *Preparation of Complementary DNA, Genomic DNA, and Sequencing*

RNA was extracted from frozen tissues by a RNA extraction kit (RNeasy Mini Kit, Qiagen, Valencia, CA). Single-strand complementary DNA (cDNA) was synthesized by AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). The double-strand cDNA was then amplified by polymerase chain reaction (PCR) and sequenced, directly or after subcloning into Bluescript I KS(–), as described.<sup>10</sup> Genomic DNA was isolated from frozen GISTs by the use of NaOH boiling preps. The genomic DNA was amplified by PCR using the forward primer (GTCGCTGTAAGATGCTCAAG) in the exon 12 and the reverse primer (TAGCAAGAGAGAACAACAGT) in the intron 13 of human *KIT* DNA, and was sequenced after subcloning into Bluescript I KS(–).

### *Construction and Transfection of Murine Mutant-Type KIT cDNA*

Generation of the murine counterpart of the human *KIT* mutant cDNA was carried out by site-directed mutagenesis as described.<sup>14</sup> Stable transfection into the interleukin-3 (IL-3)-dependent murine lymphoid Ba/F3 cell line and selection of transfectant clones by the method of limiting dilution were performed as described.<sup>10</sup> Mouse wild-type *KIT* cDNA and two mutated *KIT* cDNAs encoding well-characterized constitutively activated KIT mutants, an in-frame deletion of 6 bp encoding Val-559-Val-560 (*KIT*<sup>del559–560</sup>) in the juxtamembrane domain and a substitution of Asp-816 to Val (*KIT*<sup>816Val</sup>) in the kinase domain II respectively, were also expressed in Ba/F3 cells as controls.

### *Immunoprecipitation and Immunoblotting*

Ba/F3 clones expressing the various forms of KIT were cultivated with or without SCF (0.1  $\mu$ g/ml) for 10 minutes. Cell lysates were immunoprecipitated with rat monoclonal antibody raised against mouse KIT (clone 2B8, Pharmingen, San Diego, CA). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting was performed with anti-phosphotyrosine mouse monoclonal antibody (4G10, Transduction Laboratories, Lexington, KY) as described.<sup>14</sup>

### *In Vitro Proliferation Assay*

[3-(4,5-Dimethylthiazol-2-yl)–2, 5-diphenyl tetrazolium bromide] (MTT; Sigma, St. Louis, MO) colorimetric assay was performed as described.<sup>15</sup> Briefly, cells were cultured with various concentrations of recombinant mouse (rm) IL-3 or rmSCF for 48 hours. Cells were further cultured for 4 hours in the presence of MTT (5 mg/ml). Reaction was stopped by adjunction of acid isopropanol and the optical density at a wavelength of 540 nm was measured on a Titertek Multiskan MCC/340 (ICN Biomedicals, Costa Mesa, CA).

### *Tumorigenicity Assay in Vivo*

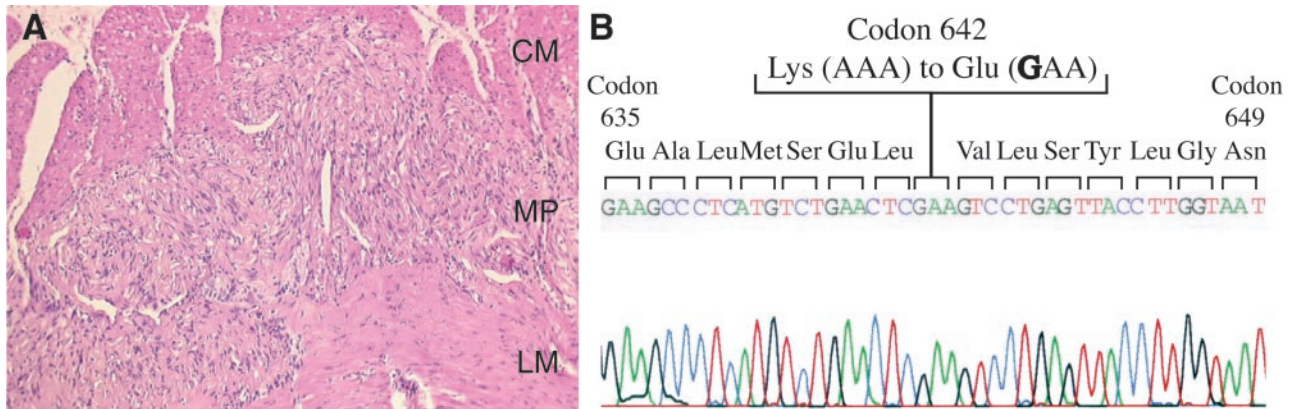
Tumorigenicity assay was performed as described<sup>15</sup> after approval by the Veterinary Committee of the Faculty of Medicine, Université Libre de Bruxelles (Brussels, Belgium). The various transfectants and original Ba/F3 cells were injected subcutaneously into the posterior flank of nude mice (Iffa-Credo, Brussels, Belgium), 5 animals in each group. The size of tumors was measured with the vernier caliper every 4 days. Tumor volume was calculated with the formula: tumor volume =  $0.5 \times a \times b^2$ , where *a* and *b* are the length and width in millimeters, respectively, of the tumoral mass. Animals were euthanized between 3 and 5 weeks after injection, depending on tumor growth rate and animal health.

## **Results**

Mother and son both showed multiple GISTs but also a diffuse hyperplasia of spindle-shaped cells in the MP region along the GI tract unaffected by GISTs (Figure 1A). GISTs, as well as ICCs in the unaffected gut, were KIT-immunoreactive (data not shown).

The whole coding region of *KIT* cDNAs was obtained from GISTs of both patients. Ten independent *KIT* cDNA clones were sequenced for each patient. A point mutation (AAA → GAA), resulting in a Lys-to-Glu substitution at codon 642 of the kinase domain I of *KIT* (*KIT*<sup>642Glu</sup>) was identified (Figure 1B). The same mutation was also present in genomic DNAs extracted from GISTs of both patients. No other mutation in the *KIT* sequence was detected. Approximately half of the cDNA and genomic DNA clones from the GISTs showed the *KIT*<sup>642Glu</sup> mutation, whereas the remaining clones showed the normal *KIT* sequence, indicating that only one allele of the *KIT* gene carried the mutation. This was confirmed by direct sequencing of reverse-transcription PCR products (data not shown). Furthermore, the *KIT*<sup>642Glu</sup> mutation, together with the wild-type *KIT* form, was also present in DNAs extracted from normal pancreas tissues from both patients, indicating the germline and heterozygous nature of the *KIT*<sup>642Glu</sup> mutant present in this family.

Activation of the KIT signaling pathway was evaluated by KIT autophosphorylation after immunoprecipitation and immunoblotting (Figure 2). In Ba/F3 cells expressing wild-type *KIT*, strong tyrosine phosphorylation of KIT occurred only after stimulation by rmSCF. In contrast, ty-



**Figure 1.** Human familial and multiple GISTs. **A:** Paraffin-embedded section (stained with hematoxylin and eosin) of intestine unaffected by GISTs, showing diffuse hyperplasia of spindle-shaped cells in MP region. Original magnification,  $\times 200$ . CM, circular muscle layer; MP, myenteric plexus; LM, longitudinal muscle layer. **B:** Mutation identified in this family: *KIT*<sup>642Glu</sup>, a substitution-type mutation at codon 642 (Lys to Glu) in the kinase domain I of *KIT*.

rosine residues of products of *KIT*<sup>del559-560</sup> and *KIT*<sup>816Val</sup> were phosphorylated even without stimulation by rmSCF, indicating the constitutive (ligand-independent) activation of these mutants. The magnitude of constitutive tyrosine phosphorylation was greater in Ba/F3 cells expressing *KIT*<sup>816Val</sup> than in cells expressing *KIT*<sup>del559-560</sup>. In Ba/F3 cells expressing *KIT*<sup>642Glu</sup>, tyrosine residues of *KIT* were also phosphorylated independently of rmSCF, at a level similar to that conferred by *KIT*<sup>del559-560</sup>.

Cell proliferation was evaluated by MTT colorimetric assay (Figure 3, A and B). Native Ba/F3 cells, which do not express *KIT*, proliferated in the presence of rmlL-3 but not with rmSCF. Ba/F3 cells transfected with the wild-type *KIT* grew in presence of either rmlL-3 or rmSCF. Ba/F3 cells expressing the constitutively activated mutants *KIT*<sup>del559-560</sup> or *KIT*<sup>816Val</sup> grew autonomously (although at different rates), even in the absence of rmlL-3 or rmSCF. Ba/F3 cells transfected with *KIT*<sup>642Glu</sup> also grew autonomously without rmlL-3 and rmSCF, at a rate comparable with that of Ba/F3 cells transfected with *KIT*<sup>del559-560</sup>.

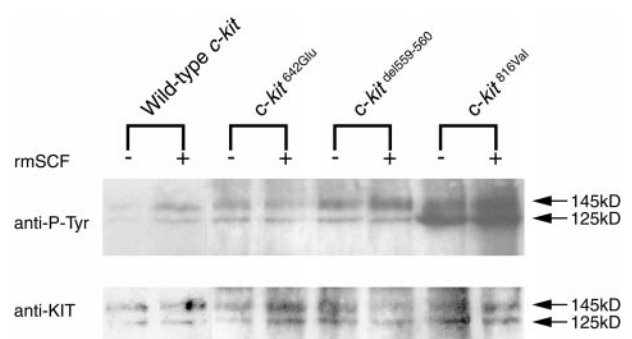
The autonomous growth conferred by expression of *KIT*<sup>642Glu</sup> in Ba/F3 cells was confirmed by the tumorigenicity assay in nude mice (Figure 3C). Original Ba/F3 cells and Ba/F3 cells expressing wild-type *KIT* did not form tumors. Ba/F3 cells expressing *KIT*<sup>del559-560</sup> or *KIT*<sup>816Val</sup> mutants caused formation of tumors in nude mice. Ba/F3 cells expressing *KIT*<sup>642Glu</sup> also resulted in tumor formation, at a rate comparable with that of Ba/F3 cells expressing *KIT*<sup>del559-560</sup>.

## Discussion

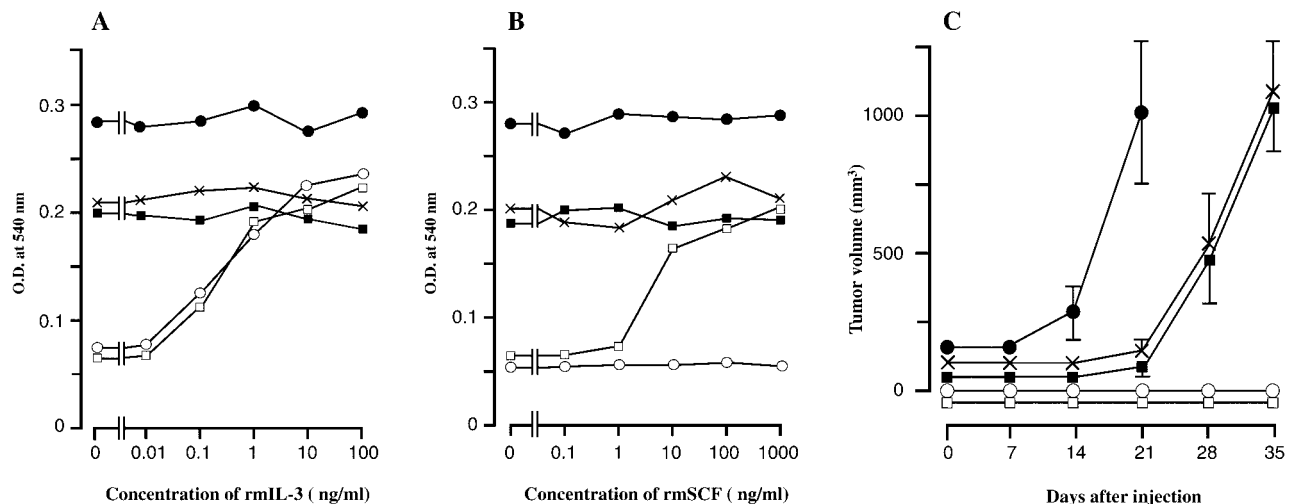
We have identified a mutation in the tyrosine kinase domain I of the *KIT* gene, *KIT*<sup>642Glu</sup>, in members of a family with multiple GISTs and demonstrated that the resulting single amino acid substitution confers a constitutive (ie, ligand-independent) activity and oncogenic properties to *KIT*. Very recently, *KIT*<sup>642Glu</sup> has been reported in two sporadic cases of GISTs.<sup>16</sup> *KIT*<sup>642Glu</sup> caused constitutive tyrosine phosphorylation of *KIT*, but biological effects were not further investigated. As the mutation was en-

countered only in tumors (ie, as a somatic mutation) and in homozygous form, it was postulated that the constitutive activity of *KIT* caused by *KIT*<sup>642Glu</sup> would be milder than that of mutations in the juxtamembrane domain and that loss of the wild-type allele would be required for tumor formation.<sup>16</sup> Conversely, our results indicate that the level of constitutive activation of *KIT* conferred by *KIT*<sup>642Glu</sup> in the Ba/F3 model is similar to that of activating mutations in the juxtamembrane domain of *KIT* previously identified in GISTs. Furthermore, the presence of a germline *KIT*<sup>642Glu</sup> of only one allele of *KIT* gene is sufficient to induce the occurrence of multiples GISTs in the proximal gut at adulthood. Germline-activating mutations in the juxtamembrane domain of *KIT* reported in other familial multiple GISTs were also heterozygous.<sup>13,17</sup>

The relationship between GISTs and ICCs has been postulated since *KIT* immunoreactivity was identified in a majority of GISTs.<sup>10,18-20</sup> The presence of *KIT* is an essential characteristics of ICCs throughout life.<sup>7,21</sup> ICCs-MP in stomach and intestine are dependant of the SCF-*KIT* signaling for their development and function.<sup>7</sup> Furthermore, differentiation and survival of embryonic



**Figure 2.** Constitutive tyrosine phosphorylation of *KIT*<sup>642Glu</sup>. In Ba/F3 cells expressing wild-type *KIT*, strong tyrosine phosphorylation of *KIT* occurred only after stimulation by rmSCF. In contrast, *KIT*<sup>del559-560</sup> and *KIT*<sup>816Val</sup> were phosphorylated even without stimulation by rmSCF, indicating the ligand-independent (constitutive) activation of these mutants. The constitutive tyrosine phosphorylation of *KIT*<sup>816Val</sup> was greater than that of *KIT*<sup>del559-560</sup>. Tyrosine residues of *KIT*<sup>642Glu</sup> were also phosphorylated independently of rmSCF, at a level similar to that of wild-type *KIT* after stimulation by rmSCF and of *KIT*<sup>del559-560</sup>.



**Figure 3.** Autonomously proliferating Ba/F3 cells expressing *KIT*<sup>G42Glu</sup> *in vitro* (A and B) and in nude mice (C). **A:** MTT colorimetric assay in the presence or absence of rmIL-3. **B:** MTT colorimetric assay in presence or absence of rmSCF. Optical density at 540 nm expressed as means of four wells. **C:** Size of tumors developed in nude mice (*n* = 5) after injection of Ba/F3 clones expressing various forms of murine KIT. Tumor volume is stated in mm<sup>3</sup> ± SE. Ba/F3 cells expressing *KIT*<sup>G42Glu</sup> showed factor-independent growth and tumorigenicity comparable to Ba/F3 cells expressing *KIT*<sup>del559-560</sup>, an activating mutation of the juxtamembrane domain found in GISTs. Ba/F3 cells expressing *KIT*<sup>S16Val</sup>, a mutant found in mast cell leukemia, showed a higher proliferation rate. Native Ba/F3 cells and Ba/F3 cells expressing wild-type *KIT* thrived only in presence of growth factors and did not form tumors in nude mice. Native Ba/F3 cells (○), Ba/F3 cells expressing wild-type *KIT* (□), Ba/F3 cells expressing *KIT*<sup>S16Val</sup> (●), Ba/F3 cells expressing *KIT*<sup>del559-560</sup> (■), Ba/F3 cells expressing *KIT*<sup>G42Glu</sup> (×).

ICCs *in vitro* also requires SCF.<sup>22</sup> Marked hyperplasia of KIT-positive cells in the MP layer of the GI tract unaffected by GISTs is a striking feature of our patients. This is reminiscent of another family with multiple GISTs, diffuse hyperplasia of KIT-positive spindle-shaped cells in the MP region<sup>23</sup> and a germline mutation in juxtamembrane domain of KIT.<sup>17</sup> It is noteworthy that in both families, hyperplasia of KIT-positive spindle-shaped cells was observed only in MP layer. KIT-positive ICCs in the deep muscular plexus layer (ICCs-DMP) appeared unaffected. In mouse, ICCs-DMP do not dependent on SCF-KIT signaling for their development.<sup>7</sup> Therefore, the presence of germline-activating mutations of *KIT* in both families indicates that constitutive activation of the KIT signaling pathway during development lead to both hyperplastic development of ICCs-MP and occurrence of GISTs in adulthood. A similar mechanism has been demonstrated for germline-activating mutations of another receptor tyrosine kinase, the proto-oncogene *c-ret*, which is involved in hyperplasia of the C cells of the thyroid gland and development of multiple endocrine neoplasia (MEN) syndromes.<sup>24,25</sup> Whether the hyperplastic cells observed in the GI tract of patients with germline-activating mutations of *KIT* gene fulfill the ultrastructural criteria of mature ICCs or of immature precursors remains to be determined but taken together, these observations favor the hypothesis of a possible common lineage for ICCs-MP and GISTs.

Intriguingly, germline-activating mutations of *KIT* apparently have little, if any, influence on melanocytes, which, like ICCs-MP, are dependent on the SCF-KIT pathway for their development.<sup>26</sup> Some hyperpigmentation of the perineum has been reported in familial GISTs with gain-of-function mutation in the juxtamembrane domain of *KIT*. This feature was attributed without investigation to hyperplasia of melanocytes.<sup>13</sup> Our patients did not show

any abnormal pigmentation. These results suggest that germline-activating mutations in the juxtamembrane domain and in the kinase domain I of *KIT* may have different transduction pathways in various cell lineages.

Activating mutations in the juxtamembrane domain of *KIT* have been previously identified in a number of KIT-positive GISTs.<sup>10</sup> Activating mutations in the juxtamembrane domain of *KIT* have been previously identified in a number of KIT-positive GISTs.<sup>10</sup> The recent identification of mutations in the extracellular domain and tyrosine kinase domain of KIT<sup>16</sup> suggests that the prevalence of *KIT* mutations in GISTs is very high and emphasizes the pivotal role of the proto-oncogene *KIT* in the ontogeny of GISTs. Mutations of *KIT* may be of prognosis value in GISTs<sup>27-29</sup> but, so far, only mutations in the juxtamembrane domain have been investigated. Reappraisal should now take into account mutations occurring in the whole coding sequence of *KIT* and their somatic or germline as well as homozygous or heterozygous nature, as these factors may differentially influence the behavior of GISTs.

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